FINAL REPORT

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Aerospace Medical Division (AFSC)
Attention: SMLPP/Plans and Programs
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CONTRACT NO: AF 41(609)-2755

TITLE OF PROJECT: Preservation of Susceptibility of Mammalian Cells to Viral Infection Following Storage in the Frozen State

REPORT PERIOD: July 10, 1965 through November 30, 1967

SUBMITTED BY: B. J. Brown, Project Director, December 15, 1967

SUMMARY

Research conducted during the period of this contract was concerned primarily with determining whether the susceptibility of mammalian cells to virus infection is altered by freezing and storage under liquid nitrogen. Although some investigations were made with so-called altered cell strains such as HeLa and KB, primary Rhesus monkey kidney cells were used as the biological system for most of this research. These primary cells were chosen because of their wide range of virus susceptibility, their relative ease of growth in vitro and the consistency of reproducibility of experimental results. The most significant findings resulting from this research were as follows:

1. Primary Rhesus monkey kidney cells may be preserved by liquid nitrogen refrigeration with an adequate per cent survival of viable and biologically

active cells, provided the initial harvest of primary cells from freshly excised kidneys were cultured in vitro before freezing.

2. The impinger vessel, developed under a previous contract (AF-41(609)-2467) was used successfully in preserving cells by liquid nitrogen refrigeration and in other experimental procedures. Although the rates of survival in liquid nitrogen refrigeration of both the altered cell strains and primary monkey kidney cells were satisfactory when the cells were frozen in impinger vessels, the results were not as good as when both types of cells were frozen in ordinary 1.2-ml ampules.
3. Following freezing, liquid nitrogen storage, and thawing, primary monkey kidney cells regularly absorbed type I poliovirus when approximately 10^6 cells were inoculated with 50-100 plaque-forming units (PFU) and incubated for as little as thirty minutes. The numbers of cells that absorbed virus increased with an increase in the length of the period of incubation and with an increase in the multiplicity of PFU in the inoculum.
4. When the multiplicity of PFU in the inoculum was at a very low level (50-100) no evidence of the release of new virus was observed below the incubation period of seven hours; however, during incubation periods of seven to twelve hours large numbers of cells were infected and the release of new virus into the culture fluid was observed repeatedly.
5. Preliminary electron microscopic observations of primary monkey kidney cells which had been infected with type I poliovirus revealed evidence of virus replication through cytological changes and the appearance of specific virus-like particles. These electron microscopic observations

were similar to those reported by earlier investigators who have studied the infection of primary monkey kidney cells with poliovirus by means of electron microscopy. Improved techniques have made it possible to observe structures in greater detail. As a consequence, we have been able to observe structures which we interpret as mature virus particles. Such particles were not observed in earlier studies with poliovirus infected primary monkey kidney cells.

6. Primary monkey kidney cells preserved in impinger vessels under liquid nitrogen refrigeration were infected when exposed to an aerosol contaminated with type I poliovirus (LSc). When assayed by the plaque technique, the titer of virus replicated by the cells were comparable to that of the original inoculum employed in the aerosol. The plaque morphology was characteristic of the LSc strain of poliovirus.
7. The experimental program conducted through the present contract has demonstrated that primary Rhesus monkey kidney cells preserved by liquid nitrogen refrigeration constitute a readily available biological system for the isolation of viral agents in aerosols.

TEXT OF REPORT

I. Introduction

Inasmuch as this is a Final Report it is intended to present, without unnecessary detail, the experimental results obtained which have contributed significant and useful information toward the objectives of the research program. In addition, the experimental data obtained since the last Progress Report (Report No. 6, dated April 21, 1967) was submitted, are included in this report.

The over-all objective of this research program has been to determine the influence of liquid nitrogen refrigeration, including freezing, storage, and thawing, on the susceptibility of mammalian cells to virus infection. There has been an awareness, also, that the primary objective of the present program is directly related to the previous research contract (AF-41(609)-2467) which involved the development and testing of a "Viral Impinger Vessel for the Storage of Mammalian Cells and Sampling of Viral Agents in Aerosols." Consequently, the experiments on virus adsorption and replication by the cells have been conducted with the lowest possible multiplicity of virus.

II. Liquid Nitrogen Refrigeration of Mammalian Cells in Ampules and Impinger Vessels

A. Experiments with Stable Cell Lines--HeLa and KB--Although this laboratory has had success with a program of long-term liquid nitrogen refrigeration of sixteen mammalian cell lines in 1.2-ml ampules (more than five years), it was considered that the impinger vessel, because of its

greater volume (approximately 30 ml) might present physical conditions which could influence adversely the rate of survival of the cells during the process of freezing and storage. Determinations were made, therefore, of viable cells of strain HeLa and strain KB which survived liquid nitrogen refrigeration when cells were frozen in impinger vessels and ordinary 1.2-ml ampules. Identical aliquots of cells from single harvests of *in vitro* cultures were frozen in impinger vessels and in ampules. After a period of liquid nitrogen storage, the cells were thawed and viability cell counts were made using trypan blue as a differential vital stain. The averages of values obtained, expressed as per cent of viable cells (number of live cells divided by the total number of cells frozen) were as follows:

Ampules		Impinger Vessels	
HeLa	KB	HeLa	KB
90.30%	85.84%	75.83%	70.00%

The above data represent the average per cent of viable cells from counts made from ten ampules and three impinger vessels for each of the two cell lines.

B. Primary Rhesus Monkey Kidney Cells Frozen in Ampules.--It has been reported that freshly trypsinized and versenated primary cultures of monkey kidney cells have been preserved successfully at -75°C (dry ice), (1,2). It was reported, also, (2) that freshly trypsinized

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(1) Stulberg, C. S., Rightsel, W. A., Page, R. H., and Berman, L. Virologic use of monkey kidney cells preserved by freezing. Proc. Soc. Exp. Biol. Med. 101:415-418 (1959).

(2) Beem, Marc O. Use of cell banked monkey kidney cells for the isolation of respiratory viruses. Bact. Proc. 1964, p. 131.

kidney cells did not survive freezing and thawing nearly as well as the versenated primary cultures. These observations have been confirmed and extended in our laboratory with liquid nitrogen refrigeration.

It is well known that the stable cell lines such as HeLa and KB withstand trypsinization with high per cent recovery of viable cells. However, when fresh monkey kidney tissue fragments are trypsinized many cells are killed, perhaps as much as 50 per cent. A comparison was made, therefore, of the rate of survival in the freezing and thawing of freshly harvested kidney cells and those that had undergone one, two, and three subcultures before freezing. In these experiments all of the cells were frozen in 1.2-ml ampules. The data obtained, Table 1, show that the rate of survival after freezing and thawing was distinctly higher when the cells were cultured than were those freshly harvested. The results of the first and second subcultures were essentially the same (84% and 83%, respectively); there was, however, an appreciable drop in the survival of cells harvested from the third subculture (76%).

Table 1. Comparative Counts of Monkey Kidney Cells Before and After Liquid Nitrogen Freezing (Trypan Blue Stain)

Type of Cell Suspension	Cell Count x 10^5		% Recovery	Average
	Frozen	Thawed		
Freshly Trypsinized Kidney	45.4	19.0	41.85	
Idem	37.0	12.9	34.85	
Idem	38.4	12.6	32.81	
Idem	42.7	14.7	34.42	35.98
First Subculture	21.2	17.8	83.96	
Idem	27.0	22.4	82.96	
Idem	27.4	22.0	80.29	
Idem	27.7	23.6	85.19	
Idem	24.5	22.0	89.79	84.44
Second Subculture	23.6	21.6	91.52	
Idem	24.4	18.0	73.77	
Idem	26.2	23.4	89.31	
Idem	19.7	15.4	78.17	
Idem	15.8	12.7	80.37	82.63
Third Subculture	38.4	29.8	77.60	
Idem	33.4	24.2	72.45	
Idem	35.4	26.2	74.01	
Idem	36.8	29.1	79.07	75.91

C. Primary Rhesus Monkey Kidney Cells Frozen in Impinger Vessels.--
After earlier experiments had demonstrated that the rate of survival of monkey kidney cells in liquid nitrogen refrigeration was greatly increased when primary cells were cultured in vitro before freezing, as compared with freshly harvested cells, this information was used to some advantage in the preservation of primary monkey kidney cells in impinger vessels. During the subsequent course of the research program Rhesus monkeys were sacrificed at varying intervals and the kidney cells which were harvested were routinely cultured in vitro. After monolayers had developed, these rapidly proliferating cells were harvested by trypsinization and aliquots of approximately 2×10^6 cells were transferred to the cell cups in a large number impinger vessels which were then subjected to the routine slow-freezing process, and refrigerated under liquid nitrogen. In this manner several batches of cells were stored in impinger vessels at different times with the result that an adequate supply of frozen cells were available for subsequent experimentation. When viable cell counts were made on the cells after thawing from thirty of these vessels, the average per cent of survival was 55.94; the highest value was 65.40 per cent and the lowest 46.79 per cent. A survival value of 50 per cent represents approximately 10^6 viable cells.

It was evident from these results with primary monkey kidney cells, as well as those obtained earlier with HeLa and KB, that mammalian cells do not survive freezing and liquid nitrogen storage in the impinger vessel as well as in the much smaller ampule. Although a

level of survival of 50 per cent is adequate for the purpose of this particular program, it is of interest to speculate regarding the difference in the results obtained with the impinger vessels as compared with the ampules. No calculated effort has been made as yet to resolve this variance. It is highly probable, however, that the difference can be attributed to the fact that the slow-freezing apparatus, being adjusted to accommodate a specific quantity of 1.2-ml ampules, does not lower the temperature at the most favorable rate when the larger impinger vessels are employed.

III. Primary Monkey Kidney Cells Infected with Virus Aerosol and Incubated without Washing

Monkey kidney cell suspensions in impinger vessels were exposed to a virus aerosol in the vacuum glove box, described previoulsy in Progress Report No. 3 (April 7, 1966). After infection, the vessels were incubated at 37°C until virus replication was complete as indicated by microscopic observation of cytopathology. The fluids in the vessels were harvested and the virus titers were determined by the plaque method on duplicate 3-oz culture bottles of primary monkey kidney cells. As a control, an aliquot of the original inoculum was titrated by the same procedure. The average plaque counts obtained during an incubation period of 6 days from the 10^{-4} and 10^{-5} dilutions of the original inoculum and the harvested fluids are presented in Table 2.

Table 2. Plaque Counts of Virus Isolated from Aerosol-Infected Monkey Kidney Cells in Impinger Vessel

Type of Inoculum	Dilution	Incubation Period (days)		
		3	5	6
Original Inoculum	10^{-4}	>100	>100	Conf
Original Inoculum	10^{-5}	43	64	Conf
Harvested Fluid	10^{-4}	83	96	Conf
Harvested Fluid	10^{-5}	3	24	27
Harvested Fluid	10^{-4}	92	>100	Conf
Harvested Fluid	10^{-5}	18	39	43

Conf = confluent

The titer of the original inoculum and the fluid harvested from the impinger vessels may be calculated as follows:

Original inoculum	$10^{7.32}$/ml (pfu)
Harvested fluid	$10^{7.13}$/ml (pfu)
Harvested fluid	$10^{7.21}$/ml (pfu)

The morphology of the plaques was essentially the same in all of the bottles and was characteristic of plaque morphology of the Lsc strain in primary monkey kidney cells under agar overlay. Also, as was expected, the titer of virus obtained by impinging the aerosol was somewhat lower than that obtained from the original inoculum. This is accounted for by the relatively smaller number of cells in the impinger vessel available to replicate virus as compared with the cells that produced virus in the original inoculum.

IV. Experimental Data Obtained Since the Last Progress Report

It was reported in Progress Report No. 6 (April 21, 1967) that primary monkey kidney cells that had been preserved in the impinger vessel under liquid nitrogen refrigeration for approximately nine months were capable of adsorbing type I poliovirus (Mahoney) when the cells were inoculated with approximately one hundred plaque-forming units (pfu) and incubated at 37°C for a maximum of thirty minutes. When the cells were washed thoroughly after the incubation to remove unadsorbed virus, approximately four per cent to eight per cent of the inoculated virus had been adsorbed as measured by plaque assay of the washed cell suspension. Moreover, as much as ninety per cent of the virus particles (pfu) inoculated were identified as being present in the infected cells and the washing supernatants. Although the per cent of virus adsorbed by the cells was relatively small, adequate evidence of virus particles having been adsorbed by the cells was obtained. There was indeed evidence of cell-virus interaction.

Subsequent to the Progress Report of April 21, additional experiments have been conducted in which considerably less than 100 pfu have been used in further investigation of the multiplicity of virus particles and the initial period of cell-virus contact required for virus adsorption and cell infection. These studies include, also, determinations of the minimum time required for the release of new virus replicated by the cells following brief exposure to low multiplicities of type I poliovirus. In addition, some preliminary investigations of the replication of poliovirus in primary monkey kidney cells by means of electron microscopy have been

carried out.

A. Infection of Primary Monkey Kidney Cells Following Liquid Nitrogen Refrigeration with Low Multiplicity of Poliovirus Particles.—A series of experiments were conducted during the period of April 15 - December 1, 1967 in which primary monkey kidney cells from twenty-one impinger vessels, which had been preserved by storage under liquid nitrogen for a period of nine to fifteen months, were inoculated with low multiplicities of type I poliovirus (Mahoney). After inoculation these samples of cells were incubated at 37°C in contact with the inoculum for periods ranging from thirty minutes to twelve hours. At the conclusion of the incubation period, the cells from each individual experiment (impinger vessel) were washed to remove completely the unadsorbed virus, and the washing supernatants and the washed cell suspensions were assayed for active poliovirus by means of the plaque technique with monolayer bottle cultures of freshly harvested primary monkey kidney cells. The procedures employed in these experiments were the same as those described in Progress Report No. 6, dated April 21, 1967.

The data obtained from the above mentioned experiments are presented in Table 3. From these data the following observations are revealed:

1. A small number of virus particles are adsorbed consistently by the cells when the incubation period (cell-virus contact) was limited to thirty minutes and when the inoculum contained as little as 50 to 100 plaque-forming units (pfu). Moreover, a high per cent of the inoculated virus was found in the washing supernatant.

Table 3. Summary of Plaque-forming Units of Type I Poliovirus (Mahoney) As Determined by Plaque Assays of Infected Primary Monkey Cells and the Supernatants Obtained from Washing the Cells

Vessel No.	Incubation Period	No. Viable Cells	PFU Inoculated	PFU in Washings	PFU in Cell Susp.	Total PFU Assayed
1	30 min	590,000	50	32	3	35
2	30 min	838,000	50	32	9	41
3	30 min	1,120,000	58	23	9	32
4	30 min	976,000	58	17	1	18
5	30 min	824,000	70	66	1	67
6	30 min	1,060,000	90	67	7	74
7	30 min	804,000	90	83	4	87
8	4 hrs	810,000	60	25	16	41
9	4 hrs	1,064,000	80	50	16	66
10	5 hrs	704,000	90	25	22	47
11	6 hrs	970,000	90	100	15	115
12	6 hrs	900,000	66	17	16	33
13	7 hrs	992,000	66	8	26	34
14	7 hrs	1,240,000	90	417	>207	>624
15	8 hrs	1,088,000	78	17	34	51
16	8 hrs	1,240,000	76	455	178	633
17	8 hrs	1,200,000	114	783	128	911
18	8 hrs	1,120,000	90	1066	130	1196
19	10 hrs	764,000	78	328	750	1078
20	10 hrs	1,152,000	76	1305	640	1945
21	12 hrs	1,060,000	78	998	708	1706

PFU--- plaque-forming units.

2. When the incubation period was between four and seven hours the number of virus particles (pfu) adsorbed by the cells was increased substantially. However, there was no indication of the release of new virus when the period of incubation was less than seven hours.

3. During incubation periods of seven to twelve hours many cells were infected with virus particles (pfu) and there was clear evidence of new virus having been released into the culture fluid.

Renato Dulbecco and his associates[1] reported the results of experiments in which 10×10^6 primary monkey kidney cells were inoculated with 1.7×10^9 plaque-forming units of type I poliovirus Brunhilde. The cells were incubated in contact with the virus inoculum at 37°C for forty minutes and then washed to remove unadsorbed virus. The washed infected cells were then incubated at 37°C for up to nine hours. During the incubation period 1 ml samples of the culture fluid from the infected cells were withdrawn for virus assay at the intervals of 0, 2, 4, 6, 7.5 and 9 hours. These samples were stored at -15°C until titrated for virus by the plaque technique on monolayers of primary monkey kidney cells. These investigators reported that the release of new virus was evident between 2 and 4 hours of incubation and reached a maximum after about 6 hours. In three experiments, an average of 86 per cent of the cells had started the "infection process" by the end of the adsorption period. The average final yield of new virus was 70 to 100 pfu per infected cell.

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[1] Frances Kallman, Robley C. Williams, Renato Dulbecco, and Marguerite Vogt. J. Biophysics and Biochem. Cytol. 4, 301-308 (1956).

In view of the fact that in our experiments approximately 10^6 cells were exposed to only 50-100 pfu, whereas Dulbecco et al employed a very much higher ratio of virus to cells (1.7×10^8 pfu to 10×10^6 cells), the results of the two experimental programs cannot be compared precisely. There is agreement, however, to the extent that the results can be compared. In both instances there was evidence of the adsorption of virus particles by the cells during the first 30 minutes of incubation. Moreover, it is reasonable to assume that the very low multiplicity of virus employed in our experiments could account for the delay in the release of new virus from 2-4 hrs, as reported by Dulbecco et al, to 7 or more hours, as shown in Table 3.

The data summarized in Table 3, as well as the data presented in previous reports, have demonstrated clearly that primary Rhesus monkey kidney cells were infected readily with type I poliovirus (LSc and Mahoney) although the cells had been subjected previously to liquid nitrogen refrigeration. It has been demonstrated, also, that such cells are capable of replicating poliovirus in essentially the same manner as freshly harvested cells.

B. Preliminary Investigation of the Replication of Poliovirus in Primary Monkey Kidney Cells by Means of Electron Microscopy.—

Methods.—After incubation periods from 2-10 hours the cells were scraped from the bottles, rinsed in phosphate buffered saline (PBS), fixed for 5 minutes in PBS containing one per cent glutaraldehyde and post fixed in one per cent tetroxide. Cells were embedded in Epon-Araldite after the normal dehydration schedule, sectioned and observed in a

Phillips 100C electron microscope.

Observations.—When studied with the electron microscope the primary monkey kidney cultures are seen to contain several cell types (e.g., epithelial, fibrocytes, fibroblasts). Many of the fibroblasts continue to synthesize collagen precursor filaments (Figure 2). Figure 1 shows a portion of two cells from a control culture.

Three hours after infection the central region of cells are filled with small membrane bound bodies (Figure 2, MB). These are probably the U bodies described by Kallman et al.[1] in their study of poliovirus infected primary kidney cultures. These structures have also been reported by other investigators[2,3] studying poliovirus infected HeLa cells. Also present at this stage of infection are large aggregates of dense material (Figure 3, VF) similar to what Dales et al. reported to be viroplastic foci.

It is difficult to correlate the profiles of the various cell types at later stages of infection. It is not clear whether this represents a difference in the sequence of events, with respect to time or susceptibility to viral infection. In any event, crystalline arrays

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[1]Kallman, F. et al., (1958) Fine Structure of Changes Produced in Cultured Cells Sampled at Specified Intervals During a Single Growth Cycle of Poliovirus. J. Biophys. Biochem Cytol. 4, 301-308.

[2]Mattern, C. F. T. and Daniel, W. A., (1965) Replication of Poliovirus in HeLa Cells: Electron Microscopic Observations. Virology 26, 646.

[3]Dales, S. et al., (1965) Electron Microscopic Study of the Formation of Poliovirus. Virology 26, 379.

as observed by Dales et al. and by Mattern and Daniel were not seen at any time from 2-10 hours post-infection in any cell type. However, in the present study some cells 7 hours post-infection contain particles in the cytoplasmic matrix (Figures 3 and 4) similar in size (260-280 A) and morphology to mature polioviruses. Kallman et al. were not able to identify any particles resembling mature viruses in their study of polio-infected cultures. In favorable regions, what appears to be empty capsids are present (Figure 4, cp).

Further study concentrating on the sequence of events in specific cell types may provide profiles in which mature viruses can be more positively identified. Such studies are also needed to test the tentative interpretation that the sequence of events leading to viral formation differs in the various cell types.

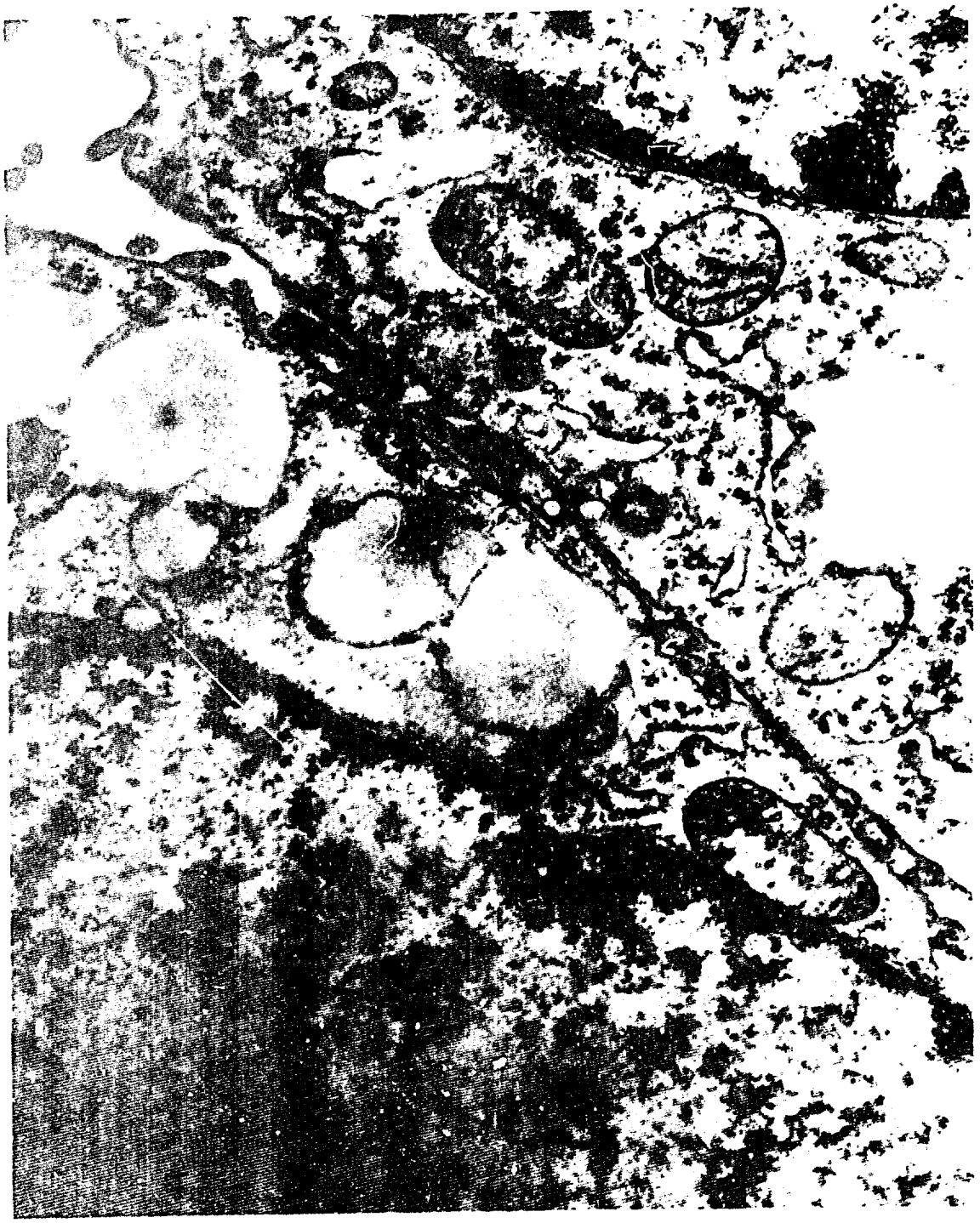

Fig. 1. Portion of two cells from a control experiment. Shows chromatin concensation, rough endoplasmic reticulum with large polysomes characteristic of fibroblasts. X 38,000.

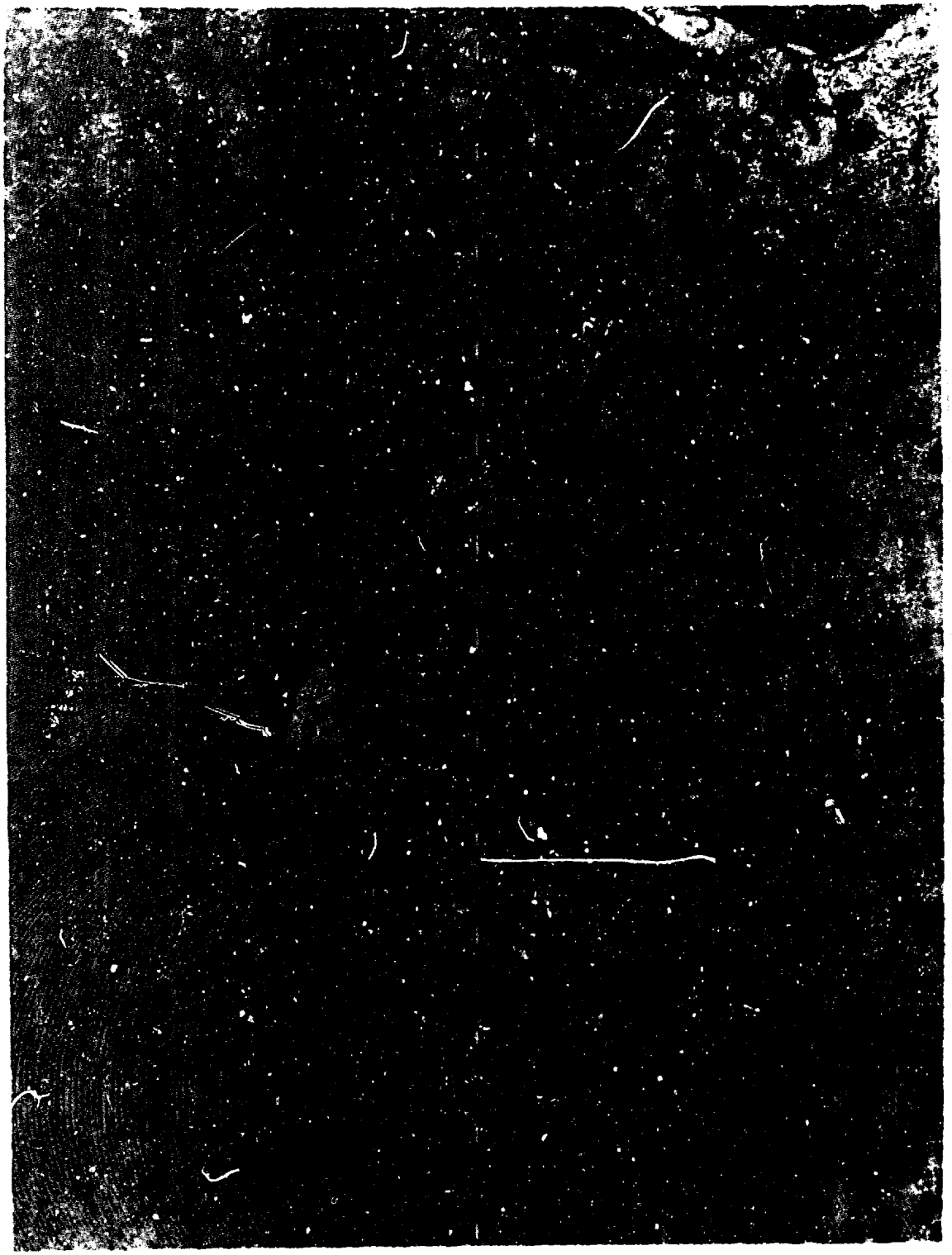

Fig. 2. Section of a fibroblast 3 hours after infection. Numerous membrane bound bodies (MB) fill the central cytoplasm along with numerous thin filaments (F) presumably precursors of collagen. Also present are dense trgious interpreted as viroplastic foci (VP). X 22,000.

Figs. 3 & 4. Portion of the outer cytoplasm of cells containing many particles (arrows) similar in size and morphology to mature poliovirus. Arrows labeled cp indicate what may be interpreted as empty viral capsids. Fig. 3, X 75,000; Fig. 4, X 55.000.